Studies on Ecology, Diversity and Phytochemical Screening of Some Freshwater Microalgae

Final Synopsis

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1. INTRODUCTION

Microalgae are a diverse group of photoautotrophs that are ubiquitous, some of which inhabit extreme ecological niches. These are primitive microorganisms with simple cell structures performing a significant role in nutrient cycling and carbon sequestering *via* photosynthesis thereby maintaining ecological balance (Shovon *et al.* 2014). As primary producers, they form the base of the food web and provide energy for all the successive trophic levels above them, therefore, they sustain life as well (Das *et al*. 2018; Saravanakumar *et al.* 2008; Tas and Gonulal 2007). They are termed as green gold of the future because of the diverse phytochemical composition that qualifies them to be used as an alternative source of food, feed and/or biofuel (Wolkers *et al.* 2011; Becker 2007).

Algae are among the fastest-growing plants in the world. This feature can be utilized to mitigate carbon dioxide emissions and also grow microalgae for biofuel (Demirbas 2009). Algae can be utilized as an integrated system for wastewater treatment, and neutral lipid production (Rodolfi *et al.* 2008; Lynch *et al.* 2015). Carbon-based compounds can be synthesized using cyanobacteria and Chlorophyceae. Their capacity to fix carbon dioxide by the process of photosynthesis and

nutritional requirements being less demanding, enable them to be used as potential raw material for biofuel production. In response to the stressful condition, algal cells accumulate neutral lipid in the form of triacylglycerols (Lynch *et al.* 2015).

To commercialize algal biofuel, algal strains with high lipid content and rapid growth rate are the prerequisites (Sharma *et al.* 2012). The need of identifying and culturing native algal species arises due to the simple fact that maintaining a monospecific culture of laboratory-selected organisms in open ponds for more than a few weeks or months is a tedious task. This is because these axenic cultures are not robust enough to withstand contamination under field conditions (Rodolfi *et al.* 2008).

Although the algal fuel seems to be a promising alternative, the net cost of production is comparably higher than the fossil oil price (Rodolfi *et al.* 2008; Pfromm *et al.* 2010). The development of efficient and commercially viable technology for microalgae-based biofuel production has been a challenging task. It is labour-intensive and time-consuming (Chen *et al.* 2011). The economic efficiency of a source for biofuel production is an important factor to be considered before suggesting an organism for biofuel production. Cell wall thickness, the presence of polyunsaturated fatty acid and cell size are important factors in determining economic efficiency (Rodolfi *et al.* 2008).

The proposed research focuses on microalgae strain selection efficient for biofuel production based on the lipid content and fatty acid profile. Also, the selection and optimization of culture conditions and growth media ideal for the maintenance and multiplication of the high lipid yielding microalgae are dealt with.

2. OBJECTIVES

1. The diversity and ecology of freshwater microalgae of lentic habitats.
2. Isolation, culture, and phytochemical screening of native freshwater microalgae.
3. Enhancement of lipid synthesis and lipid extraction from isolated microalgae.
4. Biofuel production from isolated microalgae and quality testing for its suitability as biodiesel.

Chapter 1

The diversity and ecology of microalgae of freshwater lentic habitats.

1.1 Introduction:

Algae show specificity in distribution pattern, ecology, periodicity, qualitative, and quantitative occurrence that differs widely. They prefer or tolerate specific environmental conditions (Bellinger and Sigee 2015; Celekli *et al.* 2014; Reynolds *et al.* 2002; Horne and Goldman 1994; Hall and Smol 1992). They have an innate mechanism that enables them to adapt both physiologically and morphologically to a specific environment (Reynolds 2006; Reynolds *et al.* 2002). The structural organization and species composition in an aquatic habitat vary spatially and temporally depending on the ecological conditions (Celekli *et al.* 2014). Therefore, in an aquatic habitat under a given set of environmental conditions an alga with a particular trait may dominate or co-exist (Das *et al.* 2018; Celekli *et al.* 2014; Bellinger and David 2010). Such species or communities may serve as a biological indicator of a particular habitat and perform an important function in environment management (Bellinger and Sigee 2015; Coesel 2001).

Biodiversity reinforces ecosystem stability, ecosystem services, and productivity. In recent decades, there is an unprecedented global decline in biodiversity, caused by anthropogenic factors. The bottom-up regulation by nutrients is known to be one of the key factors which impact biodiversity and ecosystem functions (Groendahl and Fink 2017). The diversity indices are not mere values but can enable an ecologist to correlate and formulate a hypothesis to understand and cautiously manage the natural ecosystem (Magurran 1983).

Many studies have investigated the effect of nutrient enrichment on the diversity of primary producers. The impact of anthropogenic activities on aquatic habitats is reflected by algal diversity and taxonomic groupings of such habitats which could be valuable environmental bioindicators (Kadam 2020; Stevenson 2014; Reynolds 1980). The species richness of the producer is known to impact standing biomass by accelerating the assimilation of inorganic nutrients. The purpose of this study was to examine the microalgal diversity and their relation to water quality in some freshwater ponds of Mangaluru, India.

2.0 Materials and methods

2.1 Description of the study site

The sampling sites (Table 1) are located between latitude 74° 49' to 75° 05' (E) and longitude 12° 44' to 13° 01(N) in proximity to the Western Ghats and are an integral part of the southwest coast of India. The climate in this region is typically monsoonal with an average annual rainfall of 3783 mm and relative humidity of ~ 88% during the monsoon. The premonsoon period i.e.

February to May is hot and humid. There is significant rainfall from June to September, followed by a short period of the post-monsoon season up to January.

Table 1: Description of study sites.

Site code	Site	Depth (m)
NK	Kalanjimale Forest	10
AMT	Amtadi, B.C. road.	8
BND	Manjalpadhe, Bondel	5
SR	Soorinje,	6
PNC	Panchalingeshwara temple pond, Urwa	4

2.2 Sampling and analysis

The study sites were chosen based on human influence on the habitat. The water samples were assessed bi-monthly from February 2016 to December 2017 from these locations. The physicochemical parameters like Temperature, Total Dissolved Solids, and Conductivity, were determined *in situ* using potable HM Digital COM - 80: EC/TDS Hydrotester and pH was measured using a portable Wasser professional pH meter. However, the other physicochemical

parameters like Dissolved Oxygen, Biological Oxygen Demand, Carbon dioxide, alkalinity, hardness, Calcium, Chloride, Nitrate, Nitrite, Phosphate, Silicate, and Sulphate were analyzed referring to APHA. The analysis was carried out in triplicates and the results were expressed as mean (± standard deviation). The temporal and spatial variations of mean physicochemical parameters were evaluated using two-way ANOVA at a 5% significance level using Prism, version 5.03 (2009).

2.3 Diversity indices

Algal sampling was done using various techniques based on their habitat. All the samples were collected in sterile specimen bottles in duplicates, from which one set of the specimen was fixed with 1% Lugol's iodine (Batram and Rees 2000) and the other set with 4% formalin (Trivedy and Goel 1986). The samples fixed in 4% formalin were assigned with a voucher number along with the date of collection and deposited at the Department of Applied Botany, Mangalore University. The organisms were identified by consulting the monographs of Coesel and Meesters (2013); Krishnamurthy (1999); Prescott (1982); Desikachary (1959); Fritsch (1948); West and West (1904, 1905, 1908, and 1912); and Ralfs (1848). The sample fixed in 1% Lugol's iodine was used for the quantitative estimation (Trivedi and Goel 1986; Bartram and Rees 2000). The abundance data were subjected to a software program PAST to determine diversity indices of the sites (Hammer *et al*. 2001).

2.4 Statistical analysis

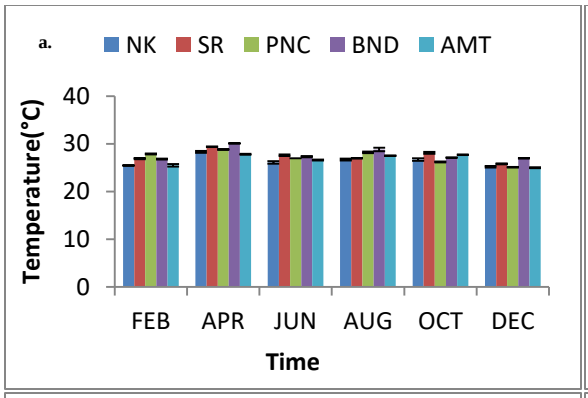
To define the significant differences between the sites, a nonparametric Kruskal Wallis test was used. The components that showed major impact were identified using principal component analysis with Kaiser normalization and Varimax rotation as dimension reducing techniques.

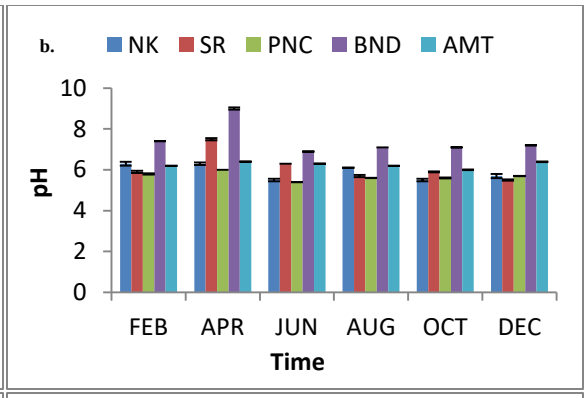
Factor analysis of species was performed to identify the principal component within the long list of phytoplankton from the five sites and species showing major impact were shortlisted. Pearson's correlation was used to evaluate the relationship between environmental parameters. Further, those components with prominent significance were utilized to perform Canonical correspondence analysis to determine the relationship, if any, between physicochemical parameters and species distribution in the study site. The data thus obtained was subjected to the forward selection of the Monte Carlo permutation test (999 unrestricted permutations).

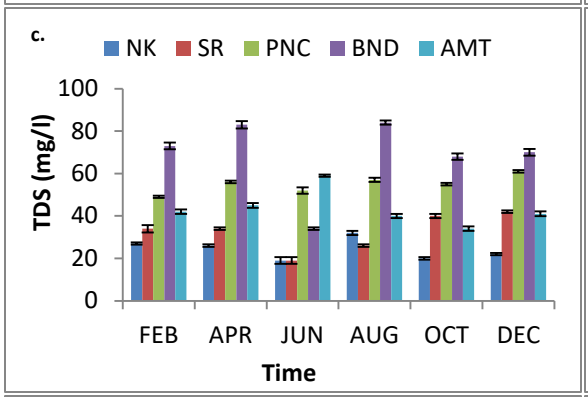
3.0 Results

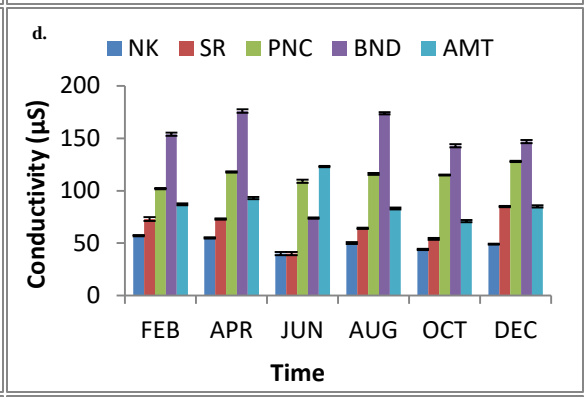
3.1 **Physicochemical parameters of the sites**

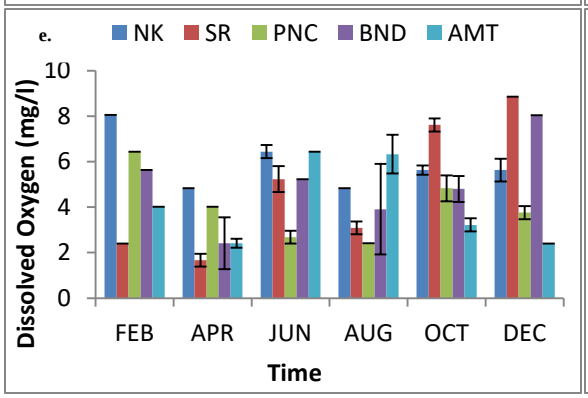
The results of the physicochemical parameters are shown in Fig (1).

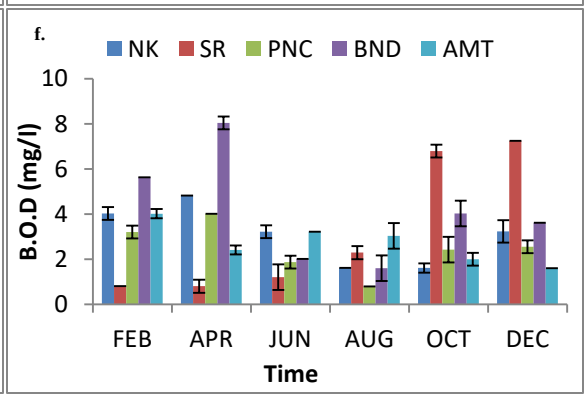

a.
NK SR PNC BND AMT
Temperature(°C)
40
30
20
10
0
FEB APR JUN AUG OCT DEC
Time

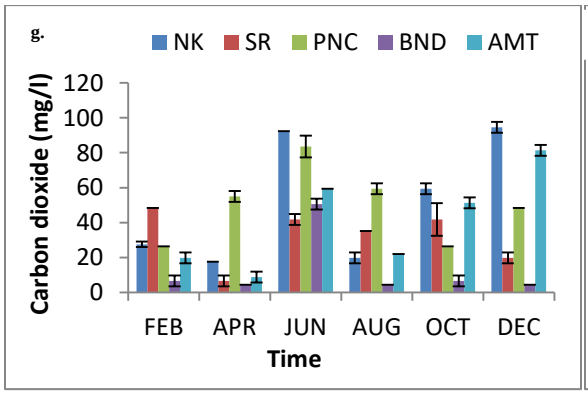

b.
NK SR PNC BND AMT
pH
10
8
6
4
2
0
FEB APR JUN AUG OCT DEC
Time

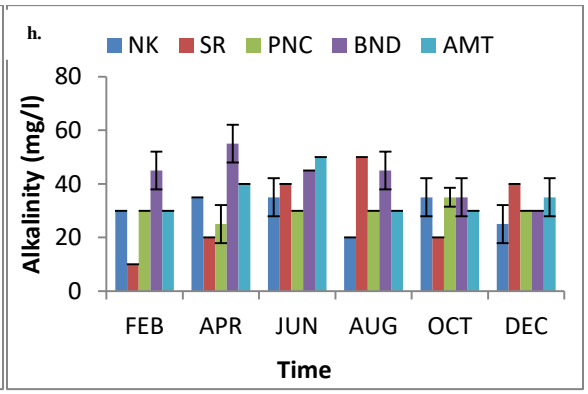

c.
NK SR PNC BND AMT
TDS (mg/l)
100
80
60
40
20
0
FEB APR JUN AUG OCT DEC
Time


d.
NK SR PNC BND AMT
Conductivity (µS)
200
150
100
50
0
FEB APR JUN AUG OCT DEC
Time


e.
NK SR PNC BND AMT
Dissolved Oxygen (mg/l)
10
8
6
4
2
0
FEB APR JUN AUG OCT DEC
Time


f.
NK SR PNC BND AMT
B.O.D (mg/l)
10
8
6
4
2
0
FEB APR JUN AUG OCT DEC
Time


g.
NK SR PNC BND AMT
Carbon dioxide (mg/l)
120
100
80
60
40
20
0
FEB APR JUN AUG OCT DEC
Time


h.
NK SR PNC BND AMT
Alkalinity (mg/l)
80
60
40
20
0
FEB APR JUN AUG OCT DEC
Time

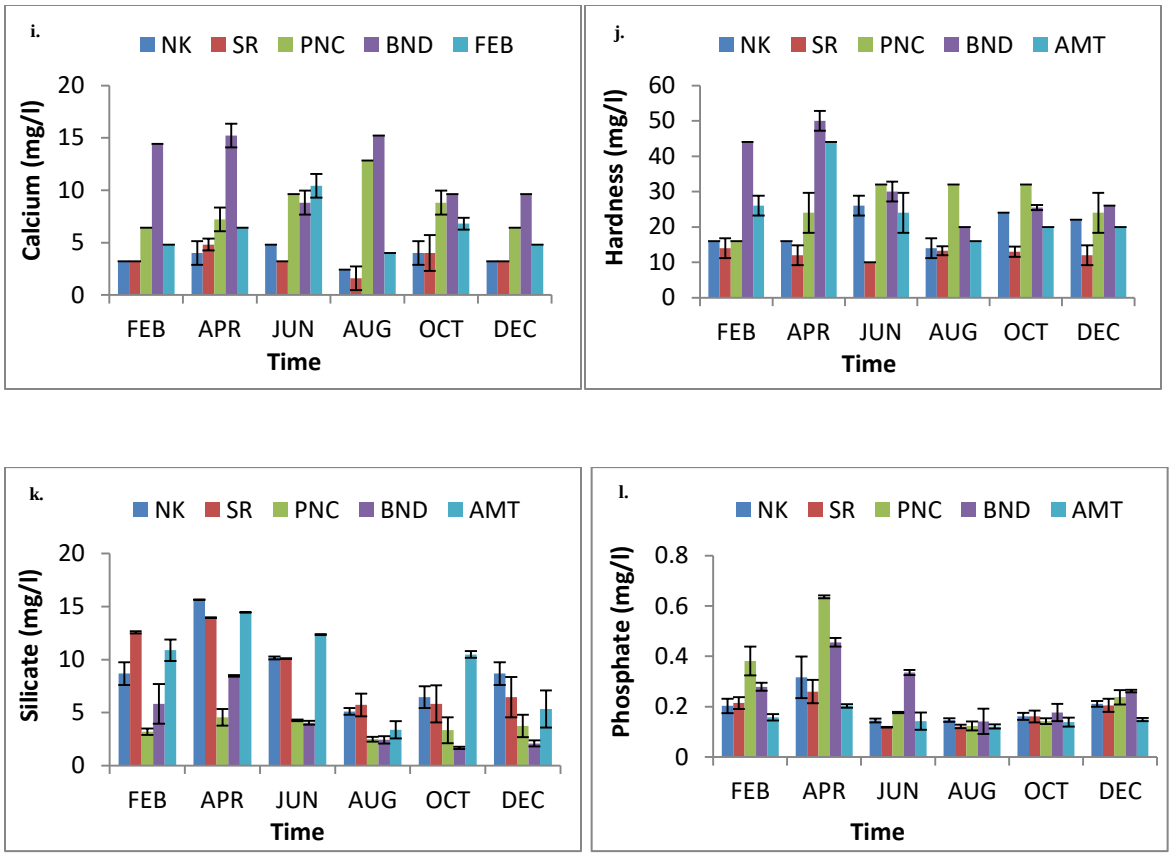
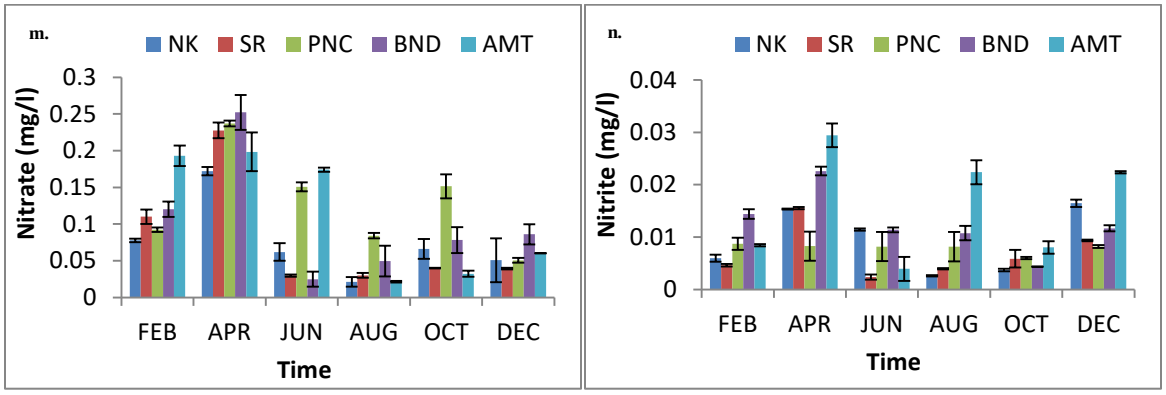


Figure 2 The physicochemical parameters of study sites during the sampling period.



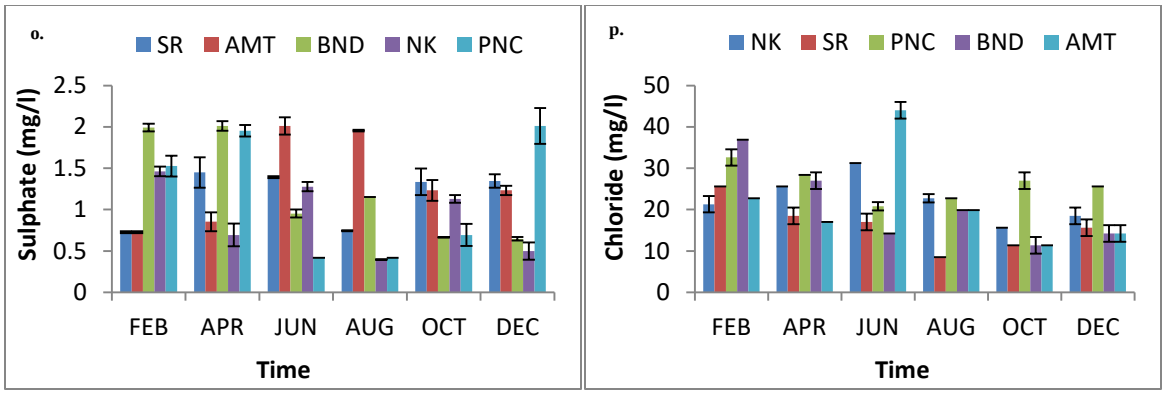


Figure 2 The physicochemical parameters of study sites during the sampling period.

3.2 Diversity of microalgae

A total of 150 taxa were found in this study belonging to seven classes i.e. Cyanophyta, Chlorophyta, Bacillariophyta, Euglenophyta, Ochrophyta, Xanthophyta and Rhodophyta. The relative numbers of species in decreasing numbers were Chlorophyceae (69.3%), Cyanophyceae (18.7%), Bacillariophyceae (6%), Eugleonophyceae (4%), Ochrophyceae (0.6%), Xanthophyceae (0.6%), and Rodhophyceae (0.6%). Chlorophyta members were dominant in all the study sites.

The Shannon's diversity (H') value ranged from 0.88 to 3.42 in all the sampling sites. The site SR showed a maximum diversity value of 3.42 during the post-monsoon season, while site PNC showed the least value of 0.88 during the pre-monsoon period. Similarly, Margalef's index of species richness ranged from 0.2 to 3.6 in the study sites. Site SR showed a maximum value of 3.6 during the post-monsoon period while the least diversity value was observed for site PNC during summer. Species richness was highest during the postmonsoon season and lowest in the premonsoon season. The dominance index ranged from 0.5 to 1.0. The highest value of

dominance was recorded during the post-monsoon period for site BND while the least dominance was observed for site AMT during the monsoon period.

3.3 Statistical analysis:

Amongst 150 species, 81 species were shortlisted as significant species from PCA, of these 17 species were Cyanophyta, 54 species were Chlorophyta, 7 species were Bacillariophyta, and 3 species were Euglenophyta. The Monte Carlo permutation test reveals that the environmental parameters linearly affect the algal species composition. The CCA analysis, covering all variables, is shown in Fig. (2).

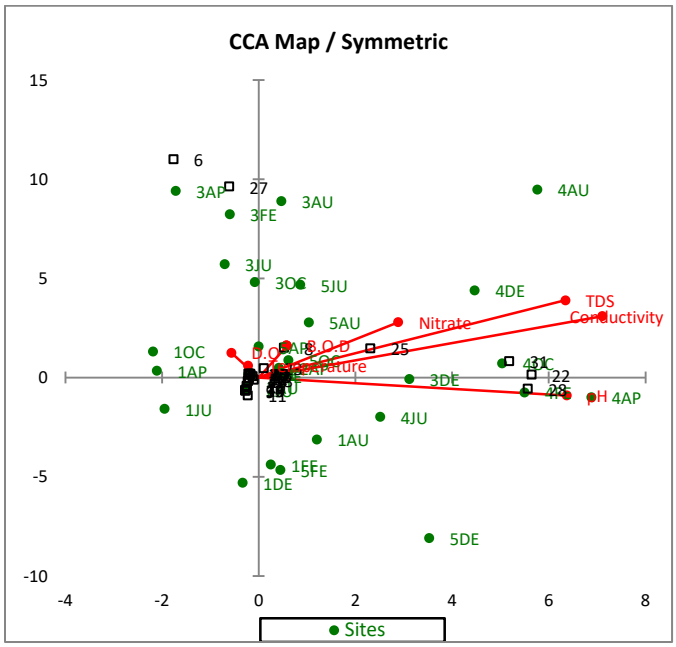


Figure 2 CCA analysis

Chapter 2

Isolation, culture and screening of native freshwater microalgae for neutral lipid production

1 Introduction

Unialgal axenic cultures are the major requirement for biochemical, physiological and genomic studies. Cultivation of microalgae *in vitro* follows most of the principles of microbial culture except for the fact that algae utilize light energy and grow autotrophically. Light, in addition to certain macro and micronutrients, needs to be supplied throughout the culture phase. The two basic criteria for successful isolation of culture are first, the media whose composition mimics the natural habitat of algae understudy and second, establishing a pure culture by eliminating all non-target species. To mimic the conditions of natural habitat it is essential to consider both the biotic and abiotic factors associated with the habitat of the target species while sampling (Mutanda *et al*. 2011).

To enhance microalgal biomass production, in addition to adequate nutrient supply, optimization of environmental factors such as pH, temperature, light intensity, and photoperiod is essential. The pH of culture media controls vital biochemical processes like solubility and availability of trace elements, speciation of inorganic sources and uptake of essential nutrients including nitrate and phosphates (Andersen 2005; Azov 1982). Though the optimum temperature of cultivation is species-dependent, a temperature range of 16-27°C is bearable by most of the algal species cultivated *in vitro*. As for higher plants, photosynthesis in algae is dependent on spectral quality, light intensity, and photoperiod. In a dense culture system, higher light intensity is preferred but, too high light intensity causes overheating and photoinhibition. The light intensities between 100

to 200 µmol photons m^{-2} sec^{-1}, corresponding to about 5 – 10% of full daylight (2000 µmol photons m^{-2} sec^{-1}) are most often employed in algal cultivation.

2. Materials and methods

All the chemicals used in media preparation were of tissue culture grade and were procured from Himedia chemicals, India. For initial isolation of microalgae half-strength BBM supplemented with soil water from respective habitats was used. Solid media was prepared by adding 1.5% agar in addition to the standard media constituents.

2.1 Isolation and identification of microalgae

The algae cells were isolated from the freshwater sample by the method described by Andersen (2005). Initial purification was done on solid Bold Basal media and BG-11 media. The morphological characters of isolated algae were studied and the species were identified referring to standard monographs. The unialgal species thus obtained were transferred to liquid media for further growth studies. The initial batch cultures of isolated algae were raised and maintained in sterilized standard media i.e. Bold Basal Media for Chlorophyceae and BG-11 for Cyanophyceae for about 30 days period at 25 ± 2°C with 12:12 of light: dark regime and irradiance of ~ 30 µmol photons m^{-2} s^{-1} provided by fluorescent light. Serial dilution and repeated subcultures were made to prevent contamination.

2.2 Screening of algae for phytochemicals

The isolated algae were subjected to initial screening to select an economically viable source. Only those species which meet the desired criteria were selected for further studies. Initial screening involved testing biomass productivity and quantitative measurement of major

phytochemicals. Isolated algae were grown in standard media for about 30 days period at 25±2°C with a light intensity of ~ 30 µmol photons m^{-2} s^{-1} and 12:12, day: dark regime. The chlorophyll-a content was estimated by the equation put forth by Mckiney (1941).

Further, the rest of the culture was freeze-dried and utilized for analysis of major phytochemical compounds. The carbohydrate content was determined using Anthrone reagent as described by Kaushik (1987). The protein content of the algal biomass was determined by the Kjeldahl method. The amino acid profile was obtained through RP-HPLC following the manufacturer's protocol (Amino acid analyser, Hitachi L-8900). Bligh and Dyer's method (1959) was used to quantify the total lipid content of the algal biomass. The general metabolite profile of methanolic extract was analyzed using HR-LCMS (QTOF). The volatile constituents in the methanolic extracts were analyzed using GC-MS.

2.3 Growth studies

Among the algae isolated initially, three species of algae namely *Chaetophora* sp., *Microchaete* sp. and *Nostoc* sp. were selected for further studies. The *Chaetophora* sp. was isolated and maintained on Bold Basal media while the Cyanophyceae *Microchaete* sp. and *Nostoc* sp. were isolated and maintained on BG-11 media. The growth of microalgae was studied in different sets by varying the composition of media, pH, temperature and light intensity. One factor at a time was used for optimization experiments. The cultures were incubated at 25°C for the desired period under ~ 30 µmol photons m^{-2} $s^{-1}$with a 12:12 day: dark regime. Effects of pH on algae were investigated by varying pH from 4 to 9 in respective media, while the effect of temperature was analyzed by growing algae under 25 °C and 30 °C. Also, the effect of light intensity was studied by incubating the cultures at 10, 30, 50 µmol photons m^{-2} s^{-1} and under outdoor

illumination (~ 428 µmol photons m^{-2} s^{-1}). The light intensity was monitored periodically using an android app Lux Light Meter pro by Doggo app, Russia. The dry weight and Chlorophyll-a content were determined at the end of incubation. Growth conditions were standardized after every set of experiment i.e., factors that showed better growth was kept constant and other environmental factors were varied accordingly.

2.4 Statistical analysis

The average values of the biomass concentration and chlorophyll-a of three replicates with their standard deviations were calculated. The Analysis of variance (ANOVA) with 95% confidence ($p < 0.05$) was used to determine significant differences. Statistical analysis was computed using Prism, version 5.03 (2009).

3 Results:

3.1 Isolation of microalgae

The isolated, cultured and identified microalgae from the freshwater habitats were *Chloroccocum* sp., *Chlorella vulgaris*, *Scenedesmus armatus*, *Chaetophora* sp., *Actinotaenium turgidus*, *Microchaete* sp., and *Nostoc* sp.

3.2 Screening of microalgae for potential phytochemicals

The yield of major phytochemicals is lower in *Actinotaenium turgidus* and *Chlorococcum* sp. Based on the yield of biomass, phytochemical constituents and ease of downstream processing *Chaetophora* sp., *Microchaete* sp., and *Nostoc* sp. were selected for further studies.

The amino acid profile of *Chaetophora* sp., *Microchaete* sp. and *Nostoc* sp shows the presence of all the essential amino acids (E.A.A). *Chaetophora* sp. contains 14 % of E.A.A, *Microchaete* sp.

contains 11% and *Nostoc* sp. contains 11%. Glutamic acid was one of the major amino acids in *Chaetophora* sp. (24%), *Microchaete* sp. (40 %) and *Nostoc* sp. (14%). HR-LCMS revealed the presence of monopalmitin, beta carotene, cholesterol and gerberinol in methanolic extracts of *Microchaete* sp and *Nostoc* sp. While in *Chaetophora* sp. several fatty acids, gabapentin, sulochrin, and fucosterol were detected through HR-LCMS. The GCMS revealed the presence of unsaturated and saturated fatty acids in all the three algae under study. The other major compounds constitute phytol, tocopherol (in *Chaetophora* sp.), gamolenic acid (in *Microchaete* sp.), neophytadiene, and stigmasterol (in *Nostoc* sp.).

3.3 Growth studies under different environmental parameters

3.3.1 pH

The algal culture grown at acidic pH showed the least Chlorophyll-a content and biomass concentration. All three species showed visible cell deformation and chlorosis when grown at acidic pH. Among the three different pH tested in the BG-11 medium, *Nostoc* sp. and *Microchaete* sp. showed better growth in pH 9.0. ANOVA showed a highly significant change in the amount of chlorophyll-a in three different pH. Further, the post-hoc test indicated a higher amount of chlorophyll-a in pH 9.0 ($p < 0.001$) compared to pH 7.0 and 4.0. While, *Chaetophora* sp., cultivated in BBM showed better growth in pH 7.0. ANOVA showed a highly significant change in the amount of chlorophyll-a in three different pH. Further, the post hoc test indicated a higher amount of chlorophyll-a in pH 7.0 ($p < 0.001$) compared to pH 9.0 and 4.0.

3.3.2 Temperature

Among the two different temperatures tested, there was higher chlorophyll-a content in the cultures grown at 25 °C. ANOVA showed a highly significant change in the amount of chlorophyll-a at two different temperatures. Further post hoc tests indicated a higher amount of chlorophyll-a in cultures grown at 25°C ($p < 0.05$) compared to 30 °C.

3.3.3 Light intensity

Among the four different light intensities tested, there was higher chlorophyll-a content in the cultures grown at an irradiance of 30 µmol photons m^{-2} sec^{-1}. ANOVA indicated a substantial change in the quantity of chlorophyll-a in the algal cells at all the four light intensities tested. Besides, the post hoc test indicated a higher amount of chlorophyll-a in cultures grown at an irradiance of 30 µmol photons m^{-2} sec^{-1} ($p < 0.05$) compared to the rest of the illumination intensity. The cultures subjected to outdoor cultivation showed reduced biomass and chlorophyll content. All three cultures showed notable morphological abnormalities when grown in outdoor conditions.

Chapter 3

Enhancement of lipid synthesis and lipid extraction

1 Introduction

The optimization of cultivation of algae, harvest, cell disruption, lipid extraction and trans-esterification of the lipids is essential for efficient biofuel production from microalgae. Solvent extraction seems to be the most viable agent to extract lipids on large scale (Chisti, 2007). Lipids can be extracted either from wet or dry algal biomass. Based on the type of biomass chosen specific cell disruption technique needs to be applied before lipid extraction. A single cell disruption technique cannot be used for a broad range of algae due to the differences in cellwall structure and organization. The composition of the cell wall varies among the different classes (also species) of microalgae (*Euglena* and *Dunaliella* sp. lack cell wall). The tensile strength of the algal cell wall (MPa) is thrice than that of the plant cell (Carpita, 1985). Strong mechanical, chemical or/and electrical forces need to be applied to rupture these tough cell wall (Lee *et al*., 2017).

Optimization of the media to enhance the lipid content of the microalgae is as important as designing an efficient lipid extraction protocol. Either of these is dependent on the species of algae under study and is crucial in determining the efficacy of the process. The biomass production and lipid accumulation in microalgae are reliant on environmental factors such as temperature, light intensity, photoperiod, pH and concentration of nutrients (like nitrates and phosphates) in the media used to cultivate them. One of the strategies used to elevate lipid content in microalgae is limiting the supply of Nitrogen sources in media. However, the main

hurdle is that nitrogen starvation would decrease the efficiency of lipid productivity and biomass content of the microalgae (Zhu *et al*. 2014).

2.0 Materials and Methods:

2.1 Lipid extraction:

The protocols of Bligh and Dyer (1959), Folch *et al*. (), and the Modified Folch's method were used for lipid extraction. Various cell disruption methods were assessed to determine higher lipid extracting procedures. The microalgal powder was subjected to grinding using mortar and pestle, homogenization, and ultrasonication. A control set without any treatment was used as a reference to determine the efficacy of the disruption technique. Lipids were extracted following Folch's method. The organic phase was pipetted out to a preweighed weighing bottle and dried. The lipid yield was calculated.

2.2 Lipid enhancement by varying nitrogen and phosphorus content:

The impact of nitrogen and phosphorus on the growth and major metabolite composition of algae was determined by growing algae in media containing 0, 50 and 100% of nitrate and phosphate in the media. The cultures were incubated at 25°C for 30 days under 3000 lux with a 12:12 day: dark regime. After the incubation period, total chlorophyll (Mckiney 1941) and major phytochemical content *viz*. carbohydrate, protein, and lipid was determined by Anthrone reagent (Kaushik 1987), Lowry's method (Lowry *et al*. 1951; Price 1965) and modified Folch's method respectively.

2.3 Statistical analysis

Data were analyzed at a 95% confidence level using one-way analysis of variance (ANOVA) with Turkey's post-hoc test and by regression analysis (Prism version 5.03, 2009).

3.0 Result:

Lipid yield was higher when algal cells were subjected to homogenization followed by extraction using modified Floch's method. Algae grown in media supplemented with 50% nitrate and phosphate content showed higher chlorophyll a. Lipid yield in *Chaetophora* sp. and *Nostoc* sp. was higher in media supplemented with 50% nitrate and phosphate ($p < 0.0001$). While lipid yield in *Microchaete* sp. was higher when cultivated in media with either nil or 50% phosphate content ($p < 0.0001$).

Chapter 4

Biofuel production from isolated microalgae and quality testing for its suitability as biodiesel.

1 Introduction:

Biofuel constitutes fatty acid methyl esters (FAMEs) derived from the transesterification of algal lipids under higher temperatures using acidified methanol (Gerpen 2005). Biofuel produced from each of the algal feedstock varies considerably in terms of fatty acid composition thereby affecting the physical and chemical properties of the biofuel. This, in turn, has a major impact on combustion characteristics and emissions from the engine (Demirbas 2005; Giakoumis *et al*., 2012). Some of the important biofuel properties that are influenced by the fatty acid profile of the feedstock are cetane number, density, kinematic viscosity, higher heating value, iodine value, cold filter plugging point, and oxidative stability. Microalgae most often contain C14:0, C16:0,

C18:0, C18:1, C18:2, and C18:3 fatty acids (Ojeda *et al.*, 2015). The selection of algal species with optimal lipid profiles has posed to be the biggest challenge in biofuel production from the algal feedstock. Here, we have checked the suitability of three microalgal feedstock for biofuel production. In particular, the fatty acid composition was studied and biodiesel properties were predicted to determine which of these would give better fuel properties with lower emissions.

2 Materials and methods:

2.1 Lipid extraction:

Lipid was extracted by the method of Nichols (1963). Further, the lipids were partitioned into chloroform by the method of Breuer *et al.*, (2013). The chloroform phase was collected into a clean glass screw cap tube, dried and stored at -20°C for further use.

2.2 Transesterification of fatty acids and profiling FAMEs:

Fatty acids were transesterified using acidified methanol followed by separation in an equal volume of n-hexane and water. The hexane phase was collected to a fresh tube and washed with water by vortexing for 5 sec followed by centrifuging at 1200 g for 5 min. The samples were stored as such at -20°C for further analysis. The n-hexane phase constitutes biodiesel. The fatty acid profile was determined by GC-MS analysis. The identification of the fatty acid esters was done by taking into account a match level of ≥ 90% with the NIST library provided with the equipment software and a peak area of ≥ 4% of the total area of each sample.

2.3 Estimation of biodiesel properties:

Biodiesel properties were estimated using the FAME profiles determined for the extracted microalgae lipids. Property data for pure fatty esters considered in the samples was calculated from correlations reported in the literature. Using this data the density at 15°C (ρ), kinematic

viscosity at 40°C (μ), higher heating value (HHV), and iodine value (IV) of biodiesel were calculated using Eq. (1) using Kay's mixing rule. The equation (1) was used to calculate the fuel properties.

$$A_b = \sum_{i=1}^{n} w_i \times f_i \qquad (1)$$

In this equation, *A* was replaced by ρ, μ, HHV or IV, depending on the property to be estimated. The subscript *i* refers to the individual i^{th} fatty ester and *b* refers to biodiesel, *n* is the number of fatty esters in the mixture, and w_i represents mass fraction. The mass fraction of individual fatty acids were calculated using Eq. (2).

$$w_i = \frac{x_i M_i}{\sum_{i=1}^{n} x_i M_i} \qquad (2)$$

Where w_i, x_i, and M_i are the mass fraction, mole fraction, and molecular weight of the i^{th} FAME, respectively (Verduzco 2013).

FAME densities (ρ) and dynamic viscosity (μ) were determined using the equation proposed by Ramirez (2013). The higher heating value (HHV) of FAMEs was determined using the equation put forth by Ramirez *et al*., 2012. Iodine value (IV) was calculated using the equation of Knothe (2002). The equation proposed by Mishra *et al*., (2016) was used to calculate the cetane number (CN) of biodiesel. Cold filter plug point (CFPP) was determined using the equation reported by Serrano *et al*., (2014). The oxidative stability (OS) was estimated by using the equation proposed by Park *et al*., (2008).

3.0 Results:

3.1 Transesterification of fatty acids and profiling FAMEs:

Fatty acids ranging from C14 to C24 were detected in FAMEs of all three algae. The FAMEs produced by *Chaetophora* sp., *Nostoc* sp., and *Microchaete* sp. is majorly comprised of C14:0, C16:0, C18:0, C18:1 and C18:2. In *Chaetophora* sp. C16-C18 constitute 66.7% of total FAMEs, while in *Nostoc* sp. and *Microchaete* sp. it constitutes 93% of total FAMEs. The C16 - C18 make up major FAMEs in algae. The PUFAs mainly consist of linolenic acid. Omega fatty acids were also detected.

3.2 Estimation of biodiesel properties:

Properties of biodiesel of *Chaetophora* sp. (BOC), *Microchaete* sp. (BOM), and *Nostoc* sp. (BON) are given in Table 1

Table 1 Predicted biodiesel properties and specifications in the U.S and European standards (EU).

Algae / Biodiesel property	BOC	BOM	BON	US	EU
CFPP (°C)	-0.79	0.33	-0.81	-	≤ 5/ ≤ -20
Dum	78.99	81.09	52.72	-	-
SCSF	149.39	173.55	161.49	-	-
CN	59.03	58.98	62.44	47[b]	51-120
OS (h)	14.47	13.45	13.75	-	-
ρ @ 15°(kg/m^3)	885.05	883.58	878.92	-	-
μ @ 40°C (mm^2/s)	3.68	3.61	3.69	1.9-6.0	3.5-5.0
HHV (MJ/kg)	39.60	39.59	39.70	-	-
IV (g I /100g biodiesel)	132.79	119.94	82.86	-	120[a]

Highlights

- **Microalgae play a significant role in nutrient cycling and fixing inorganic carbon and thereby help to mitigate carbon dioxide. T**he concentration and composition of the cellular components like protein, lipids, carbohydrates etc. of the algae are significantly influenced by the ecological condition where it thrives. The algal cells have more efficient access to nutrients in an aquatic ecosystem therefore slight changes in the habitat structure would be reflected in terms of their species composition and cellular constituents. Therefore understanding habitat composition enables one to formulate precise media and environmental conditions for successful *in vitro* cultivation of algae from such aquatic ecosystems for sustainable commercial applications.
- *Chaetophora* sp. was cultured in Bold Basal media with pH 7 and incubated at 25 ± 2°C with an irradiance of 30 µmol photons m^{-2} sec^{-1} and 12:12 light: dark regime, while, the Cyanophyceae was cultured in BG-11 media with pH 9.0 and incubated at 25 ± 2°C with an irradiance of 30 µmol photons m^{-2} $sec^{-1}$and 12:12 light: dark regime.
- Various cell disruption techniques were applied to obtain better lipid yield. Microwave and sonication were found to be efficient in extracting maximum lipid from the *Chaetophora* sp., *Microchaete* sp. and *Nostoc* sp. Microwave extraction followed by the modified Folch's method is more economical (less time consuming) for lipid extraction from lyophilized samples than other techniques. Media supplemented with 50% nitrate and phosphate content is preferable for cultivating *Chaetophora* sp., *Microchaete* sp. and *Nostoc* sp. for biofuel as the algae shows optimum biomass and lipid productivity in it.

- Properties of biodiesel of *Chaetophora* sp., *Microchaete* sp., and *Nostoc* sp. follow both European and US standards for biodiesel. Of the three, *Nostoc* sp., would be potential biodiesel feedstock.

List of the paper published:

Shetty, K., & Krishnakumar, G. (2020). Algal and cyanobacterial biomass as potential dye biodecolorizing material: a review. *Biotechnology Letters*, *42*(12), 2467-2488.

Shetty, K., & Gulimane, K. (2022). Biomonitoring of freshwater lentic habitats using desmids. *Limnology*, *23*(1), 245-251.

List of conferences attended:

- "**Enhancement of primary metabolite production in *Chaetophora* sp. by varying media constituents**". International Webinar on Recent Trends in Autotrophic Cryptogam Research - 7 - 8 October, 2021. Organised by Department of Ecology & Environmental Science, Assam University, Silchar, CSIR-National Botanical Research Institute, Lucknow and Indian Lichenological Society (ILS), Lucknow
- "**Diversity and Seasonal variation of a Pond in Mangalore, Karnataka**" - Bioprospecting of Algae: Resources Conservation and Utilization (National conference) - 1 & 2 August 2019 - Department of Plant Sciences, Central University of Kerala.

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